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Did Trypanosomatid Parasites Contain a Eukaryotic Alga–Derived Plastid in Their Evolutionary Past?

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ABSTRACT: The Trypanosomatidae is closely related to euglenids that harbor plastids acquired from a green alga via secondary endosymbiosis. This discovery led to the idea that trypanosomatid parasites contained a green alga–derived plastid in their evolutionary past, an evolutionary scenario that was criticized based on the rarity of plant/plastid/cyanobacterium-like genes in the completely sequenced genomes of *Trypanosoma* and *Leishmania* species. Because it is difficult to identify such genes, however, their apparent rarity does not preclude a previous plastid endosymbiosis in the Trypanosomatidae. The genome of the plastid-less apicomplexan *Cryptosporidium parvum* preserves only a handful of plant/plastid/cyanobacterium-like genes, suggesting massive loss of plastid genes after elimination of its plastid. Additional support for such wholesale gene loss comes from fucoxanthin-containing dinoflagellates. Trypanosomatid nuclear genomes contain cyanobacterium-, green plant–, and haptophyte alga–derived genes, suggesting that they could have possessed a plastid in their evolutionary past; however, these genes also could represent examples of more typical horizontal gene transfer that did not accompany a plastid endosymbiosis. Thus, the presence of host cell genes that were adapted for use in the plastid would be much stronger evidence for a past plastid endosymbiosis in the Trypanosomatidae. Good examples of such genes are those encoding superoxide dismutases (SODs). Trypanosomatid parasites possess 4 iron-containing SODs, with 2 of them, SODA and SODC, targeted to the mitochondrion. In contrast with SODAs with classical single-domain mitochondrial targeting signals, SODCs carry bipartite pre-sequences composed of a signal peptide, followed by a transit peptide. Interestingly, these N-terminal extensions show striking similarities in length, hydropathy profiles, amino acid composition, and targeting properties to pre-sequences of proteins targeted to eukaryotic alga–derived plastids of euglenids and dinoflagellates. In turn, phylogenetic analyses indicate that SODCs originated from a mitochondrion-targeted SOD via gene duplication and were inherited vertically in the trypanosomatid lineage. These data represent a new kind of evidence for a past plastid endosymbiosis in the Trypanosomatidae, but the nature of this plastid remains unclear. It is usually assumed that the trypanosomatid plastid shared a common origin with that of euglenids, but Δ4 desaturase phylogenies suggest that it could have originated via an independent, tertiary endosymbiosis involving a haptophyte alga. It is also possible that ancestors of the Trypanosomatidae initially possessed a primary plastid that later was replaced by a secondary or tertiary plastid.

BACKGROUND

The Trypanosomatidae is a group of parasitic protozoans that includes medically important species, such as *Trypanosoma brucei* (the causative agent of African sleeping sickness), *T. cruzi* (Chagas' disease), and *Leishmania donovani* (Kala Azar or visceral leishmaniasis) (for a recent review, see Simpson et al., 2006). The common feature of their cells is the presence of peculiar mitochondrial DNA known as the kinetoplast, composed of 2 kinds of circular molecules encoding proteins and guide RNAs (gRNAs) (Lukeš et al., 2005). Trypanosomatid parasites also possess modified peroxisomes called glycosomes, which are involved both in β-oxidation of fatty acids and biosynthesis of ether lipids, as well as in glycolysis (Michels et al., 2006). Further notable features of their cells are: (1) import of numerous tRNAs from the cytosol into the mitochondrion (Yermovsky-Kammerer and Hajduk, 1999); (2) an unusual uridylate insertion/deletion type of RNA editing in mitochondrial transcripts (Lukeš et al., 2005); (3) *trans*-splicing of cytosolic pre-mRNAs (Liang et al., 2003); and (4) the presence of base J in nuclear DNA (Borst and van Leeuwen, 1997).

Trypanosomatids are very closely related to bodonids and diplonemids (Simpson and Roger, 2004a; Von der Heyden et al., 2004). Bodonids include free-living bacterivores and micro-predators, as well as ecto- and endoparasitic forms, whereas diplonemids are represented by free-living, surface-associated heterotrophs and occasional facultative parasites. Interestingly, the sister group to the trypanosomatid, bodonid, and diplonemid assemblage is the Euglenoidea (Simpson and Roger, 2004a; Von der Heyden et al., 2004). Euglenids comprise both heterotrophic and photosynthetic species; a typical representative of their photosynthetic forms is *Euglena gracilis*. *Euglena* plastids are surrounded by 3 membranes, contain chlorophylls *a* and *b*, and have evolved from a green alga (Gibbs, 1978; Sulli et al., 1999; Takahashi et al., 2007). Given the close evolutionary relationship between trypanosomatids and euglenids, it has been hypothesized that the Trypanosomatidae contained a plastid in their evolutionary past (Hannaert et al., 2003).

Trypanosomatid, bodonid, diplonemid, and euglenid lineages together form the phylum Euglenozoa (Cavalier-Smith, 1993). Bleached mutants of *E. gracilis* provide a mechanistic model of plastid loss within this phylum. Although the heterotrophic euglenid *Astasia* (*Euglena*) *longa* maintains a vestigial non-photosynthetic plastid with reduced plastid DNA (ptDNA) (Gockel and Hachtel, 2000; Krajčovič et al., 2002), it appears that plastids of *E. gracilis* can be lost. Different physical and chemical factors, e.g., elevated temperature, UV irradiation, and the antibiotic streptomycin, result in the so-called bleaching of *E. gracilis* cells (for reviews, see Bodył, 1996; Krajčovič et al., 2002). During this process, ptDNA can be degraded or even lost (Heizmann et al., 1982; Hussein et al., 1982; Conkling et al., 1993); however, this is neither sublethal nor lethal, suggesting that *E. gracilis* is on the way toward losing its plastids. In support of this hypothesis, one of the bleached strains of *E. gracilis*, W10BSmL, was found to be devoid of any plastid (Osafune and Schiff, 1983).

EVOLUTIONARY ORIGIN OF THE HYPOTHETICAL TRYPANOSOMATID PLASTID: PRIMARY, SECONDARY, OR TERTIARY ENDOSYMBIOSIS?

Modern plastids originated by 3 kinds of endosymbiotic events, i.e., primary, secondary, and tertiary endosymbiosis (for reviews, see Palmer, 2003; Archibald, 2009). In primary endosymbiosis, plastids evolve directly from cyanobacteria, in secondary, from algae with primary plastids, and tertiary, from algae with secondary plastids. More details related to plastid evolution are included in Figure 1. Below, we discuss the evolutionary origin of the hypothetical trypanosomatid plastid in the context of known variations among plastid endosymbioses.

Primary endosymbiosis

Plastids derived from cyanobacteria, or primary plastids, are known from glaucophytes, red algae, green algae, and higher plants (Palmer, 2003; Archibald, 2009). It is widely accepted that these algae and plants constitute the kingdom Plantae (=Archaeplastida) and that their common ancestor enslaved of a cyanobacterium (Deschamps et al., 2008). However, phylogenetic analyses by Nozaki et al. (2003, 2009) demonstrated paraphyly of the kingdom Plantae, with red algae diverging early in eukaryotic evolution and green algae representing a sister group to either the Euglenozoa or the Chromalveolata. Based on these data, they proposed

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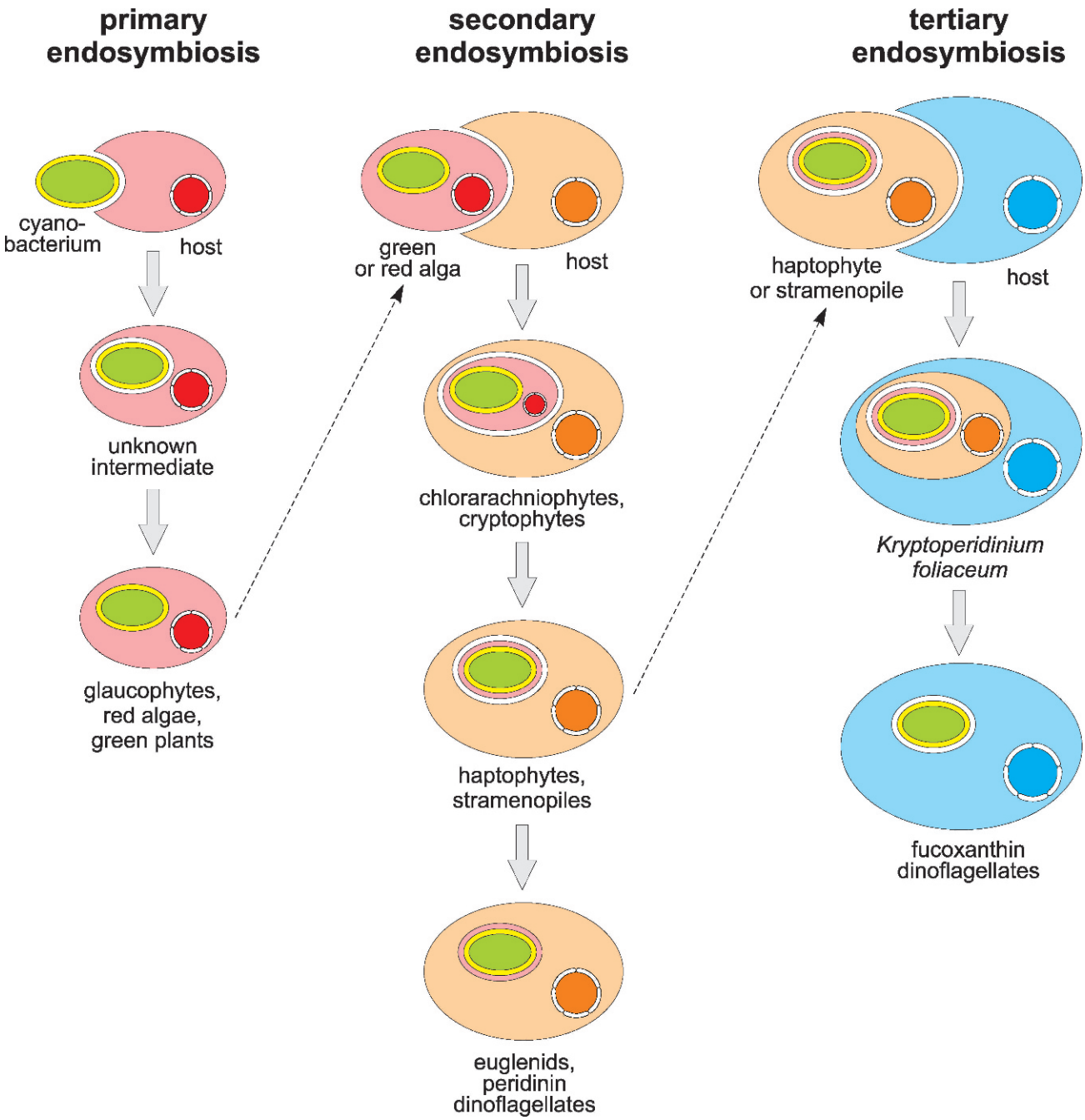

FIGURE 1. Three pathways of plastid acquisitions by eukaryotic cells. In primary endosymbiosis, a eukaryotic host engulfs a cyanobacterium, which then is converted into a plastid surrounded by 2 membranes. Both of these membranes are derived from the plasmalemma and the outer membrane of the cyanobacterial endosymbiont (Cavalier-Smith, 2003). An intermediate stage in the evolution of primary plastids is an alga with plastids surrounded by 3 membranes, with the outermost one originating from the host endomembrane system. This membrane was lost in glaucophytes, red algae, and green plants. In secondary endosymbiosis, a heterotrophic eukaryote acquires a plastid from a green or red alga. Such a plastid originally is surrounded by 4 membranes: the innermost 2 originate from the endosymbiont plastid, the third is derived from the primary endosymbiont's plasmalemma, whereas the outermost membrane represents the secondary host's phagosomal membrane (Cavalier-Smith, 2003). Chlorarachniophytes and cryptophytes represent an intermediate stage in the evolution of secondary plastids, because their plastids still maintain cytoplasm and the nucleus (now the nucleomorph) of the eukaryotic algal endosymbiont (Archibald, 2007). The Haptophyta and Stramenopila lost the nucleomorph, whereas peridinin dinoflagellates and euglenids lost both the nucleomorph and one of the envelope membranes. In tertiary endosymbiosis, a eukaryotic host enslaves a stramenopile or haptophyte, which is finally transformed into a plastid surrounded by 3 membranes. Tertiary plastids with 3 envelope membranes are characteristic for fucoxanthin dinoflagellates. It is likely that their 2 innermost membranes correspond to the envelope of primary plastids, whereas the outermost membrane is derived from the host endomembrane system (Bodył and Moszczyński, 2006). Fucoxanthin plastids evolved from a haptophyte alga. An intermediate stage in the evolution of tertiary plastids is represented by the dinoflagellate *Kryptoperidinium foliaceum*, whose stramenopile-derived plastids still possess 5 membranes, the nucleus, and the cytoplasm with mitochondria. The membrane surrounding the stramenopile endosymbiont represents its plasmalemma, which means that the phagosomal membrane was lost (McEwan and Keeling, 2004). Please note that this scheme focuses on the main stages in the conversion of endosymbionts into plastids, not on evolutionary relationships between different groups of eukaryotic algae.

a new concept of Plantae, wherein ''plants'' are defined not only as algae with primary plastids, but also as a diverse assembly of forms apparently derived from such algae. These relatives include both protozoans with no plastids, e.g., ciliates, katablepharids, and foraminiferans, and algae with eukaryotic alga–derived plastids, e.g, cryptophytes, stramenopila (=heterokonts), dinoflagellates, and apicomplexans (see below). Thus, in the Nozaki et al. model, eukaryotes acquired a primary plastid very early that later was lost from many protist lineages, including trypanosomatid parasites (Fig. 2C) (see also Nozaki, 2005; Maruyama et al., 2009).

Concordant with this evolutionary scenario, Opperdoes and Michels (2007) identified several cyanobacterium-derived genes in trypanosomatid nuclear genomes. These include 6-phosphogluconate dehydrogenase, adenylate kinase, fructose-1, 6-bisphosphate aldolase, and trypanothione reductase. Another peculiar case includes 6 genes encoding the pyrimidine biosynthetic pathway, which are organized in a pattern identical to that of their cyanobacterial homologs (Opperdoes and Michels, 2007). These findings are consistent with the proposal that trypanosomatids (or their ancestors) contained a primary plastid in their evolutionary past. Additional support for such an ancient primary endosymbiosis comes from the recent phylogenetic studies of Rogozin et al. (2009). They found that the first bifurcation on the eukaryotic tree is that between Plantae and the remaining eukaryotic groups (including Excavata, chromalveolates, Amoebozoa, opisthokonts), suggesting that all eukaryotes could have evolved from a plastid-containing ancestor. Consistent with this view, Yuan et al. (2007, 2008) identified possible footprints of an ancient cyanobacterial endosymbiosis in animals and fungi.

Secondary endosymbiosis

Plastids derived from algae with primary plastids (i.e., secondary plastids) are found in many algal lineages, such as dinoflagellates, Cryptophyta, stramenopiles, Haptophyta, chlorarachniophytes, and, most notably, the euglenids (Palmer, 2003; Archibald, 2009). Hannaert et al. (2003) published several phylogenetic trees of nuclear-encoded genes on which trypanosomatid sequences clustered with those of plants and cyanobacteria. Considering the close evolutionary relationship between trypanosomatids and euglenids, they proposed that the Trypanosomatidae previously contained a green alga–derived plastid that was lost with the adoption of a parasitic lifestyle (Fig. 2A) (see also Martin and Borst, 2003). Although some of the phylogenetic trees published by Hannaert et al. (2003) were called into question because of a poor taxon sampling (Rogers and Keeling, 2004), phylogenetic analyses of YCF45s seem to provide new support for a past green algal endosymbiosis in these parasites (Opperdoes and Michels, 2007). We will subsequently consider this issue in more detail.

Tertiary endosymbiosis

Plastids derived from algae with secondary plastids (i.e., tertiary plastids) are characteristic for several dinoflagellate lineages (McEwan and Keeling, 2004; Bodył and Moszczyński, 2006; Patron et al., 2006). One of these lineages is represented by the dinoflagellates *Kryptoperidininum foliaceum* and *Durinskia baltica*, which acquired their plastids from a stramenopile alga (McEwan and Keeling, 2004). Trypanosomatid parasites possess a biosynthesis pathway for polyunsaturated fatty acids, which includes the enzyme Δ4 desaturase. Interestingly, on phylogenetic trees of this protein, *Trypanosoma* and *Leishmania* Δ4 desaturases cluster with sequences from haptophytes and stramenopiles (Tripodi et al., 2006). As already mentioned, Haptophyta and Stramenopila contain complex plastids that evolved from a red alga via secondary endosymbiosis. Thus, the acquisition of Δ4 desaturases by trypanosomatids would have been possible via temporal or permanent tertiary endosymbiosis with a haptophyte alga (Fig. 2D). In support of such an evolutionary scenario are 2 further examples of gene transfer from eukaryotic algae with red plastids to euglenids; the first is represented by 6-phosphogluconate dehydrogenase (Maruyama et al., 2008) and the second by triosephosphate isomerase (Sun et al., 2008).

The ''replacement'' versus ''shopping bag'' model

Available data suggest that the hypothetical trypanosomatid plastid could have evolved from either a cyanobacterial or an algal endosymbiont, but these scenarios are not mutually exclusive. It is possible that ancestors of the Trypanosomatidae initially contained a primary plastid that later was replaced by a secondary or tertiary plastid (Häuber at al., 1994). Such evolution via plastid replacement was recently argued for diatoms (belonging to Stramenopila) although, in this case, the replaced plastid was proposed to have a secondary green algal origin (Moustafa et al., 2009). A ''replacement'' model can explain the chimeric nature of the trypanosomatid nuclear genome, but we should consider alternative evolutionary scenarios as well. For example, the symbiogenetic origin of cell organelles is usually characterized as a process involving a single host enslaving a single endosymbiont. It is also plausible, however, that the ancestors of such a host maintained numerous transient relationships with distinct endosymbionts as described by the ''shopping bag'' hypothesis (Larkum et al., 2007). Numerous genes from each of these endosymbionts would be transferred to the host nucleus, enabling establishment of a true cell organelle. A good example of such evolution is the chlorarachniophyte *Bigelowiella natans* (Archibald et al., 2003). Thus, it is possible that trypanosomatids could have harbored only 1 type of plastid, whereas some cyanobacterial/algal genes were derived from temporary endosymbionts.

YCF45 AND A PAST PLASTID ENDOSYMBIOSIS IN THE TRYPANOSOMATIDAE

Trypanosomatid parasites possess a YCF45 protein (Opperdoes and Michels, 2007). This protein is encoded in the nucleus and contains AAA-type ATPase- and cytochrome P450–like domains; however, its function currently is unknown. In red algae and algae with secondary red plastids (such as haptophytes and stramenopiles), YCF45 is encoded by plastid DNA, whereas in green plants, the gene was transferred to the nucleus (Opperdoes and Michels, 2007). The latter authors suggested that this protein was acquired by trypanosomatid parasites via horizontal gene transfer from either a cyanobacterium or a plastid. In support of this hypothesis, they adduced a phylogenetic tree on which the sequences from *Leishmania* and *Trypanosoma* species group with those of green plants.

Both *Leishmania* and *Trypanosoma* YCF45s carry an N-terminal extension that probably functions as a targeting signal. Our bioinformatic analyses using 6 programs that predict targeting signals and intracellular localizations of a given protein, i.e., iPSORT (Bannai et al., 2002), TargetLOC (Hoglund et al., 2006), Predotar (Small et al., 2004), PredSL (www.195.134.85.247/PredSL/index.html), PProwler (Bodén and Hawkins, 2005), and TargetP (Emanuelsson et al., 2000), indicate that all of these extensions have characteristics typical of mitochondrial targeting signals. This suggests that trypanosomatid YCF45s are imported into mitochondria. Although Opperdoes and Michels (2007) assumed that YCF45s of green plants carry typical plastid transit peptides, our in silico investigations did not provide full support for this assumption. We found that only 42% of plant sequences were equipped with plastid transit peptides, whereas 31% had mitochondrial transit peptides. This indicates that plant YCF45s might be imported into both plastids and mitochondria.

We could assume that green plant and trypanosomatid YCF45s were inherited vertically from an ancestral primary plastid. After its loss from trypanosomatids, the hypothetical plastid-targeted YCF45 would have been re-directed to the mitochondrion. This scenario is compatible with the phylogentic trees of Nozaki et al. (2003) on which green plants cluster with the Euglenozoa. However, on some recent global phylogenies of Eukaryota, almost all photosynthetic eukaryotes group together, forming a mega-assemblage (Burki et al., 2008, 2009). The only exception is euglenids, which branch outside this clade and cluster with the Trypanosomatidae, Heterolobosea, jakobids, and *Histions* spp. (see also Nozaki et al., 2009). Such a topology suggests a horizontal transfer of YCF45 from a green alga to trypanosomatids, perhaps via a secondary plastid endosymbiosis. However, targeting signals of proteins imported into euglenid plastids, which are derived from a green alga (Takahashi et al., 2007), carry bipartite and tripartite pre-sequences (Durnford and Gray, 2006). Consequently, single-domain pre-sequences of trypanosomatid YCF45s do not provide clear support for a green algal plastid endosymbiosis. We could hypothesize that their pre-sequences were initially bipartite (or tripartite) and that, after plastid elimination, they were converted to mitochondrial targeting signals by a loss of the signal peptide domain. However, plant YCF45s also carry mitochondrial transit peptides, and it is possible that one such gene was transferred to the Trypanosomatidae without a green algal plastid endosymbiosis. Conse-

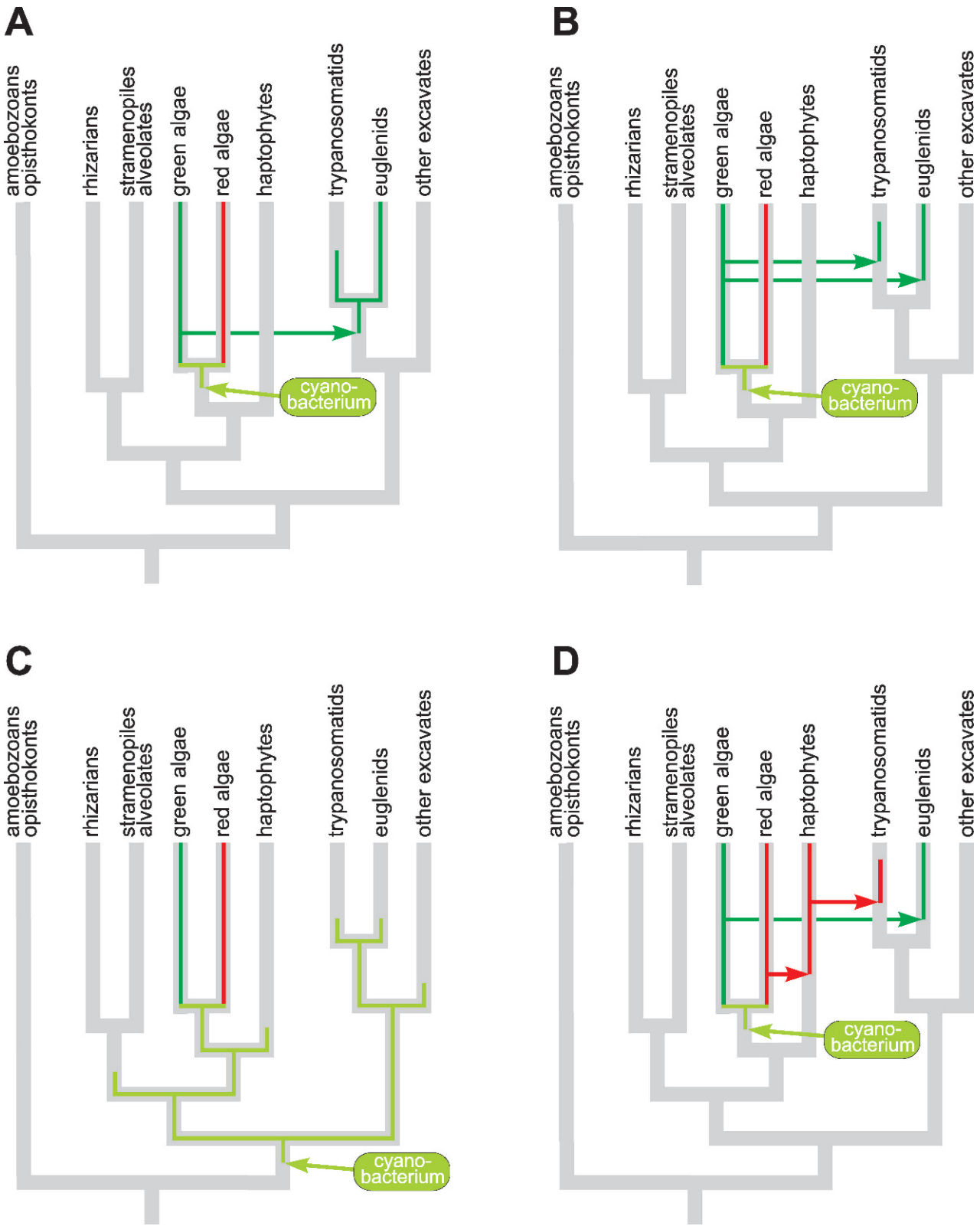

FIGURE 2. Four possible scenarios to explain the evolutionary origin of the hypothetical trypanosomatid plastid. (**A**) The classical model postulates a common origin of the trypanosomatid and euglenid plastids from a green alga via secondary endosymbiosis. (**B**) An alternative secondary scenario also assumes a green algal origin of the trypanosomatid plastid, but independent from that of euglenids. (**C**) It is possible that ancestors of the Trypanosomatidae contained a cyanobacterium-derived plastid, acquired very early in eukaryotic evolution via primary endosymbiosis but lost subsequently on multiple occasions. (**D**) The trypanosomatid plastid could also have evolved from a haptophyte alga via tertiary endosymbiosis. Hypotheses B–D assume independent origins of trypanosomatid and euglenid plastids. Evolutionary relationships between selected groups of eukaryotes are based on the phylogenetic trees published by Burki et al. (2008) and Okamoto et al. (2009).

quently, the YCF45-based evidence for a past green algal endosymbiosis in trypanosomatids needs further investigation.

WHY DO TRYPANOSOMATID GENOMES CONTAIN SUCH FEW PLANT/PLASTID/CYANOBACTERIUM-DERIVED GENES?

It is usually assumed that if a eukaryotic lineage contained a plastid in its evolutionary past, it should preserve footprints of such an endosymbiosis in the form of plastid genes localized in the host nucleus (see, for example, Palmer et al., 2004). However, the completely sequenced genomes of 3 trypanosomatid species, *Leishmania major*, *T. brucei*, and *T. cruzi*, are characterized by the rarity of plant/plastid/cyanobacterium-like genes (El-Sayed et al., 2005). Consequently, the latter authors questioned whether the Trypanosomatidae contained a plastid in their evolutionary past (see also Leander, 2004). It should be noted, however, that difficulties with identification of plant/plastid/cyanobacterium-like genes in trypanosomatid parasites are the expectation, even if an endosymbiont had been present in their past. After loss of photosynthesis, almost all plastid-related genes would have been lost as well; any that remained could have undergone such significant divergence that their endosymbiotic ancestry would be difficult to recognize.

There is a model for this process in the Apicomplexa. This is a large taxon embracing parasitic protozoans, e.g., *Plasmodium falciparum* and *Toxoplasma gondii*, in which a non-photosynthetic plastid, called the apicoplast, was found (for a review, see Waller and McFadden, 2005). There also are aplastidic apicomplexans, however, such as *Cryptosporidium parvum* (Zhu et al., 2000). The recent discovery of *Chromera velia*, which seems to be a photosynthetic relative of the Apicomplexa (Moore et al., 2008), suggests that progenitors of *C. parvum* once possessed a plastid that was lost entirely (Zhu et al., 2000; see also Toso and Omoto, 2007). However, Huang et al. (2004) found only several genes that represent footprints of a plastid endosymbiotic ancestor in the nuclear genome of *C. parvum*.

Additional support for the potential of massive plastid gene loss from trypanosomatid parasites comes from dinoflagellates. Most photosynthetic dinoflagellates possess a peridinin plastid surrounded by 3 membranes, which contains a peculiar genome composed of plasmid-like DNA molecules (or minicircles) and encoding only a handful of plastid proteins (for reviews, see Bodył and Moszczyński, 2006; Howe et al., 2008). Consequently, almost all plastid proteins in peridinin dinoflagellates are encoded by the host nucleus. These proteins carry complex presequences composed of 2 or 3 distinct domains and are targeted to the plastid through the endoplasmic reticulum (ER) and/or Golgi apparatus (Patron et al., 2005; Bodył and Moszczyński, 2006). Interestingly, in some dinoflagellate lineages, the peridinin plastid was replaced by plastids acquired from distinct algal sources. For example, fucoxanthin dinoflagellates, such as *Karlodinium micrum* or *Karenia brevis*, acquired their new plastids from a haptophyte alga (Yoon et al., 2005; Patron et al., 2006). Because these plastids resemble the peridinin plastid in the number of envelope membranes and the structure of targeting signals (Bodył and Moszczyński, 2006; Patron et al., 2006), it was initially presumed that *Karlodinium* and *Karenia* plastids used the ER-Golgi–based targeting machinery and hundreds of nuclear-encoded plastid genes from the original peridinin plastid, thereby simplifying conversion of the engulfed haptophyte algae into fully integrated organelles (Cavalier-Smith, 2003). However, Patron et al. (2006) found very few such genes in *K. micrum*, and Yoon et al. (2005) were unable to identify any in *K. brevis*. Therefore, there was massive, or perhaps complete, loss of identifiable nuclear genes associated with the peridinin plastid after it was replaced by the haptophyte-derived plastid in fucoxanthin dinoflagellates. With respect to peridinin plastid–related genes, this process appears to have transformed *Karlodinium* and *Karenia* genomes almost back to the ancestral heterotrophic state, before acquisition of the original peridinin plastid (Yoon et al., 2005). An analogous reversion could have occurred in parasitic trypanosomatids after loss of a plastid, particularly given their highly modified lifestyle.

Ciliates are heterotrophic protists devoid of any kind of plastid, including a non-photosynthetic one. Along with dinoflagellates and apicomplexans, they constitute the super-group Alveolata (Fast et al., 2001) that is suggested to have once contained a plastid (Cavalier-Smith, 2003). In support of this view, Reyes-Prieto et al. (2008) identified 16 algal-derived genes in the completely sequenced genomes of 2 ciliates, *Tetrahymena thermophila* and *Paramecium tetraurelia*. If ciliates actually harbored a plastid in their evolutionary past, its elimination would have to be accompanied by the loss of hundreds of its nuclear-encoded genes.

Indirect evidence for plastid gene loss in the Trypanosomatidae is provided by mitosome-containing protozoans. Mitosomes are highly reduced mitochondria serving only as sites for biosynthesis of Fe-S clusters (for a review, see van der Giezen et al., 2005). These organelles were found, for example, in the diplomonad *Giardia lamblia* (Tovar et al., 2003). Analyses of the *G. lamblia* nuclear genome demonstrated that it is devoid of almost all typical mitochondrial genes, e.g., those responsible for aerobic and anaerobic ATP synthesis, with the exception of the genes encoding proteins involved in the Fe-S cluster formation (Morrison et al., 2007).

GENOMIC EVIDENCE FOR A PAST PLASTID ENDOSYMBIOSIS: ENDOSYMBIONT- VERSUS HOST-DERIVED GENES

The extensive genomic studies of Reyes-Prieto et al. (2008) suggest that ciliates once possessed a plastid, but these data might also be explained by multiple horizontal gene transfers, as was clearly stated by these authors (see also the ''you are what you eat'' hypothesis; Doolittle, 1998). In support of this latter view, multiple horizontal gene transfers from bacteria were demonstrated for anaerobic ciliates inhabiting the foregut of ruminants (Ricard et al., 2006). Moreover, *Paramecium* spp. harbor green algal endosymbionts (Kodama and Fujishima, 2008) and it is very likely that they donated/donate numerous genes to these ciliates' nuclei (see also Archibald, 2008). It also is possible that the several alga- or cyanobacterium-derived genes, or both, in the nuclear genome of *C. parvum* (see the previous section) might be the result of multiple horizontal gene transfers. The *Chromera* plastid is usually regarded as a vertically inherited photosynthetic ancestor of the apicoplast (Moore et al., 2008), but we should consider an alternative scenario in which these plastids originated by independent endosymbiotic events (Bodył et al., 2009). Thus, cryptosporidians, along with gregarines, would represent primitively heterotrophic, aplastidal lineages.

The genomic data from ciliates and cryptosporidians have important implications for searching for evidence of a past plastid endosymbiosis in trypanosomatid parasites. Even if trypanosomatid nuclear genomes are found to possess many plant/plastid/cyanobacterium-like genes in future studies, we cannot definitively exclude that these represent examples of horizontal gene transfer without plastid acquisition. It is well known that some trypanosomatids, e.g., *Phytomonas* species, parasitize plants (Pappas et al., 2005), whereas others, e.g., *Crithidia deanei* or *Herpetomonas roitmani*, possess bacterial endosymbionts (de Souza and Motta, 1999). These and other tightly associated organisms could be easy sources of plant/plastid/cyanobacterium-like genes for trypanosomatid genomes. Therefore, plastid-related genes that originated via duplication within the host nucleus and were inherited vertically rather than horizontally would be much stronger support for a past plastid endosymbiosis in the Trypanosomatidae. Examples of such unique duplication events are well known and have been suggested for cytosolic genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Harper and Keeling, 2003) and fructose-1, 6-bisphosphate aldolase (FAB) (Patron et al., 2004) in a putative common ancestor of chromalveolate algae (Takishita et al., 2009). After these duplications, one of the genes was free to acquire an appropriate targeting pre-sequence and the encoded protein then could be imported into the plastid.

SUPEROXIDE DISMUTASES PROVIDE EVIDENCE FOR A PAST PLASTID ENDOSYMBIOSIS IN THE TRYPANOSOMATIDAE

Analogous evidence for a past plastid endosymbiosis in trypanosomatids is found in SODs (Bodył and Mackiewicz, 2008), which catalyze the disproportionation of superoxide radicals to hydrogen peroxide and oxygen and represent a first line of defense against reactive oxygen species (ROS) (for a review, see Scandalios, 2005). Trypanosomatid parasites encode as many as 4 iron-containing superoxide dismutases (Dufernez et al., 2006; Wilkinson et al., 2006). One of them resides in the cytosol and a second is imported into peroxisomes/glycosomes. The remaining 2, designated as SODA and SODC, reside in the mitochondrion (Dufernez

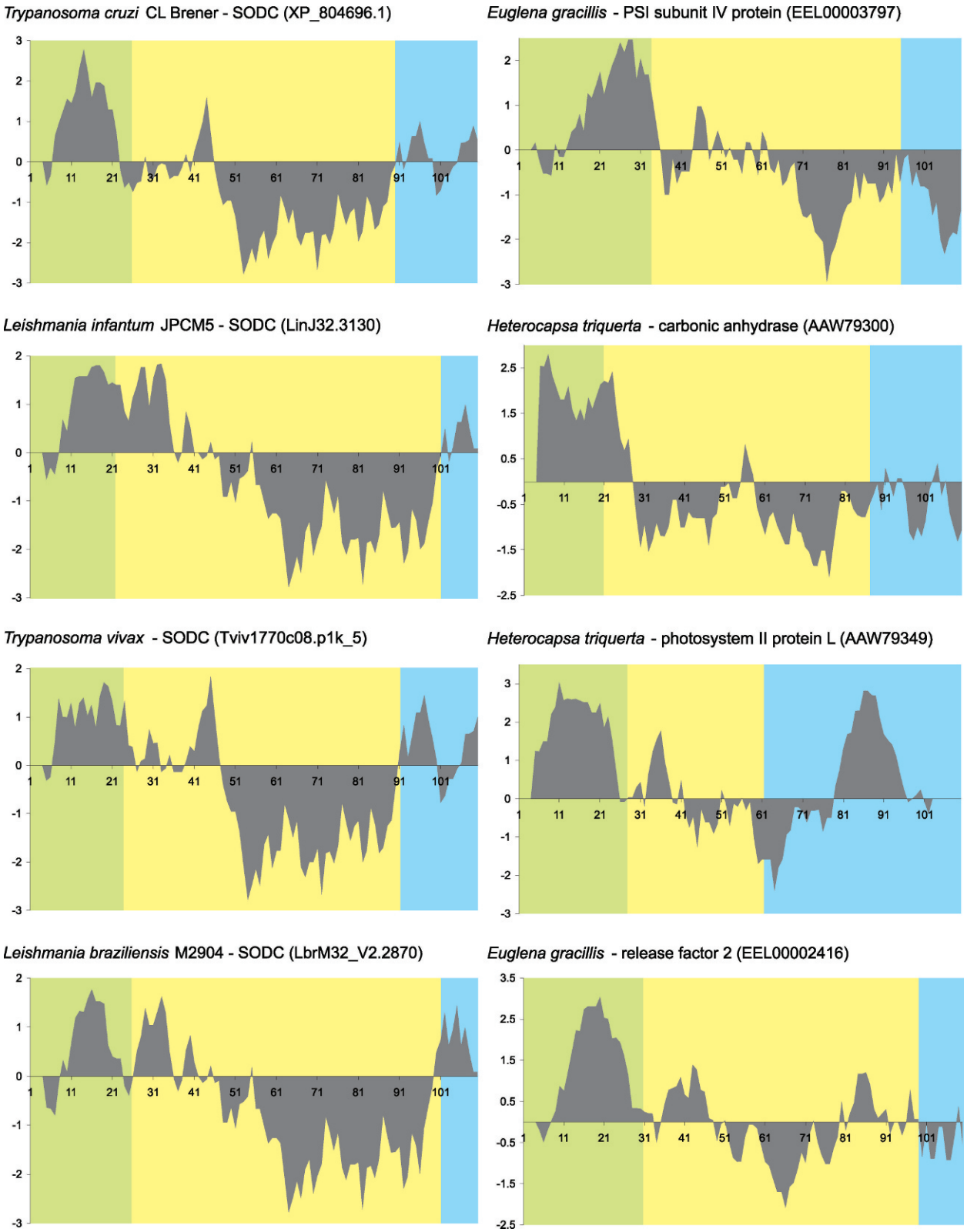

FIGURE 3. Comparison of hydropathy profiles of N-terminal extensions of trypanosomatid mitochondrion-targeted SODCs with those of proteins imported into multimembrane plastids of euglenids and dinoflagellates. *Leishmania* and *Trypanosoma* SODCs are assembled in the first column. Please compare hydropathy profiles of their pre-sequences with those of plastid proteins of euglenids (*Euglena gracilis*) and dinoflagellates (*Heterocapsa triquetra*) presented in the right column. The green region corresponds to the hydrophobic domain (functioning as a signal peptide), the yellow to the hydrophilic domain (acting as a transit peptide), and the blue to the mature protein. Hydropathy profiles were made according to the Kyte-Doolittle scale (Kyte and Doolittle, 1982), assuming a sliding window length of 9 residues. The hydropathy values are represented in the y axis, whereas values in the x axis correspond to position in analyzed sequence. Signal peptides and their cleavage sites were predicted by SignalP (Bendsten et al., 2004).

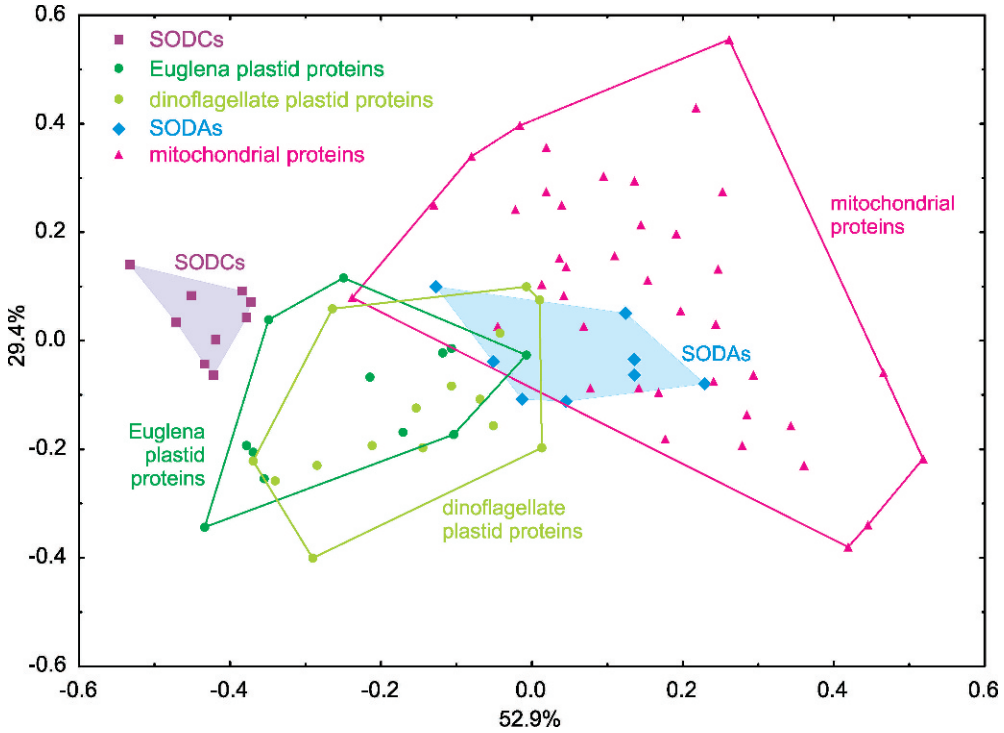

FIGURE 4. Results of correspondence analysis of amino acid content of pre-sequences of SODCs, plastid-imported proteins of euglenids and dinoflagellates, SODAs, and trypanosomatid mitochondrion-targeted proteins. These analyses were performed on 4 physicochemical classes of amino acids: acidic (D, E), basic (H, K, R), polar (N, Q, S, T, Y), and hydrophobic or nonpolar (A, C, F, G, I, L, M, P, V, W). SODCs are clearly separated from SODAs and other mitochondrial proteins, and are most similar to plastid proteins. The x and y axes correspond to the first and the second principal component, respectively. The values at these axes indicate the percentages of variance explained by each of these principal components.

et al., 2006; Wilkinson et al., 2006). Although SODAs and SODCs are delivered to the same organelle, they differ drastically in length and structure of their N-terminal extensions. SODAs carry typical 1-domain mitochondrial transit peptides consisting of ~30 amino acids (Dufernez et al., 2006; Wilkinson et al., 2006; Getachew and Gedamu, 2007; Bodył and Mackiewicz, 2008). In contrast to these dismutases, SODC pre-sequences are composed of ~100 amino acids and have hydrophobic domains at their N-terminals. These domains were recognized as signal peptides by computer programs, such as TargetP (Emanuelsson et al., 2000), SignalP (Bendsten et al., 2004), iPSORT (Bannai et al., 2002), Phobius (Käll et al., 2004), and Predotar (Small et al., 2004) (see Dufernez et al., 2006; Wilkinson et al., 2006; Bodył and Mackiewicz, 2008).

The bipartite nature of N-terminal extensions of SODCs, a hydrophobic domain followed by a hydrophilic domain (Bodył and Mackiewicz, 2008), is identical to pre-sequence structure of proteins targeted to eukaryotic alga–derived plastids (Fig. 3) (for reviews, see Ishida, 2005; Hempel et al., 2007). In the algal N-terminal extensions, the first (hydrophobic) domain is characteristic of a signal peptide, whereas the second (hydrophilic) domain is a feature of transit peptides. Their remarkably similar structures suggest that N-terminal extensions of SODCs functioned previously as targeting signals that rendered their import into a secondary (or tertiary) plastid surrounded by 3 or 4 membranes. In support of this view, there are striking similarities in length, hydropathy profiles, amino acid composition, and targeting properties of SODC pre-sequences and those of proteins targeted to multimembrane plastids of *Euglena* spp. and dinoflagellates (Fig. 3) (Bodył and Mackiewicz, 2008). These similarities are seen especially well in correspondence analysis of amino acid composition of the pre-sequences of SODCs, SODAs, and trypanosomatid mitochondrial proteins, as well as euglenid and dinoflagellate plastid proteins (Fig. 4).

It is likely that trypanosomatid parasites initially possessed only 1 mitochondrion-directed SOD, with a classic single-domain mitochondrial targeting signal (Fig. 5) (see also Bodył and Mackiewicz, 2008). After (or just before) acquisition of the secondary or tertiary plastid, this dismutase gene underwent duplication, giving rise to SODA and SODC. Originally, both dismutases would have been imported into the mitochondrion, but acquisition of a signal peptide by SODC enabled its import into the eukaryotic alga–derived plastid (Fig. 5). It is reasonable to assume that the hypothetical trypanosomatid plastid initially was photosynthetic and generated large amounts of ROS that were regularly scavenged by the plastid-directed SODC. With adoption of a parasitic lifestyle, the plastid probably lost its ability to photosynthesize, resulting in a drastic decrease in the production of ROS (Fig. 5). Consequently, mutations that rendered re-targeting of SODC to the mitochondrion were favored by selection.

In accordance with predictions of this model, apicomplexan parasites possess a Fe-SOD that carries a plastid-like bipartite pre-sequence composed of a signal peptide, followed by a transit peptide (Brydges and Carruthers, 2003; Pino et al., 2007). This dismutase is dually targeted to the apicomplexan mitochondrion and its complex plastid, surrounded by 3 or 4 membranes (Pino et al., 2007). By analogy with the apicomplexan dismutase, it could be hypothesized that the trypanosomatid SODC also was initially dually targeted to both the plastid and the mitochondrion but, after plastid elimination, it is delivered only to the mitochondrion.

Additional support for our evolutionary scenario comes from phylogenetic trees of iron-containing superoxide dismutases. These trees show that SODCs are sister to SODAs (Dufernez et al., 2006), indicating that they are paralogs and were duplicated early in trypanosomatid evolution (Fig. 5). Moreover, SODAs and SODCs cluster with the dismutases of parabasalids (Dufernez et al., 2006); these protists, like trypanosomatids, belong to the super-assemblage Excavata (for reviews, see Simpson and Roger, 2004b; Keeling et al., 2005). This suggests that the gene duplication leading to SODA and SODC paralogs in trypanosomatids occurred after they diverged from euglenids, at which point a secondary or tertiary plastid could have been present in the Trypanosomatidae.

SUB-MITOCHONDRIAL LOCALIZATION OF SODCS: MITOCHONDRIAL MATRIX, INTERMEMBRANE SPACE, OR BOTH OF THESE COMPARTMENTS?

Considering the serious differences in length and structure of SODA and SODC pre-sequences, Dufernez et al. (2006) suggested that these

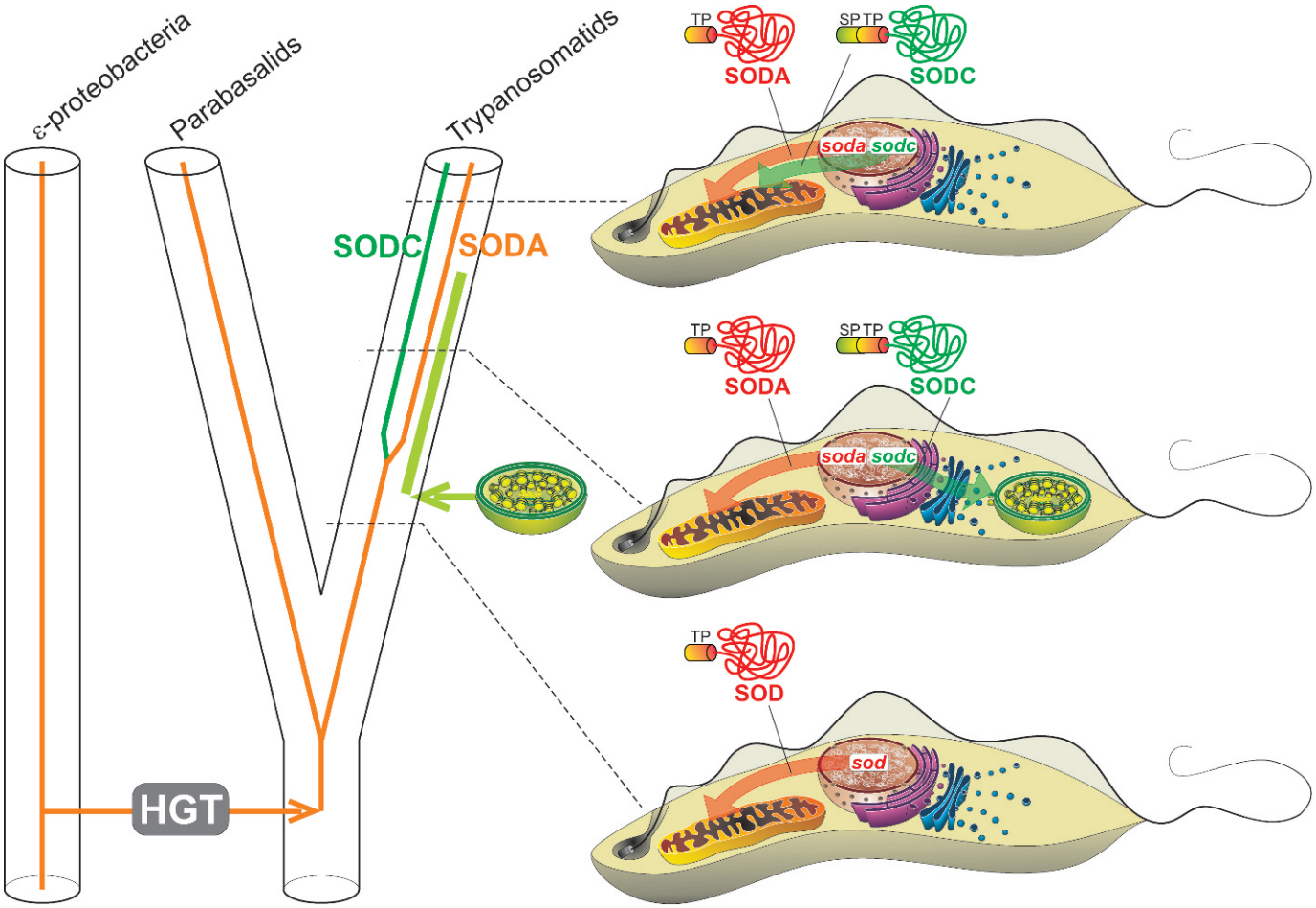

FIGURE 5. Evolution of SODCs and their targeting within the trypanosomatid cell. SODCs currently are targeted to the mitochondrion, but they carry bipartite, plastid-like pre-sequences composed of a signal peptide (SP), followed by a transit peptide (TP). On the phylogenetic tree of Fe-containing superoxide dismutases, SODCs cluster with trypanosomatid SODAs that also are imported into the mitochondrion, but possess typical mitochondrial targeting signals, with only 1 domain corresponding to the plastid transit peptide (Dufernez et al., 2006; Bodył and Mackiewicz, 2008). This suggests that SODCs originated by duplication of a *sod* gene encoding a mitochondrion-targeted protein in the trypanosomatid host cell lineage. A sister group for the SODC plus SODA clade comprises superoxide dismutases of the parabasalids *Trichomonas vaginalis* and *Tritrichomonas foetus* (Dufernez et al., 2006). Parabasalids and trypanosomatids belong to the super-assemblage Excavata, indicating that SODCs and SODAs were inherited vertically, not horizontally. It is possible, however, that the ancestor of Excavata acquired a *sod* gene from a ε-proteobacterium via a horizontal gene transfer (HGT), because on the SOD tree, trichomonads and trypanosomatids group with *Wolinella*, *Campylobacter*, and *Helicobacter* bacteria (Dufernez et al., 2006). We suggest that a heterotrophic ancestor of the Trypanosomatidae originally possessed a single gene encoding a mitochondrion-targeted SOD, which carried a classical mitochondrial-targeting signal. Before or after acquisition of a eukaryotic alga–derived plastid, this gene underwent duplication, giving rise to SODA and SODC. Both dismutases initially were imported into the mitochondrion, but the incidental gain of a signal peptide by SODC, e.g., via exon shuffling, made its targeting to the plastid possible. After plastid loss, this dismutase was re-directed to the mitochondrion. Selection then favored modifications of the SODC bipartite pre-sequence that facilitated its function as a single domain mitochondrial-targeting signal, especially in its signal peptide.

dismutases are targeted to distinct mitochondrial sub-compartments. In this model, SODAs are transported into the mitochondrial matrix, whereas the destination place for SODCs is the intermembrane space. The matrix residence of SODAs has been well documented by several independent studies (Dufernez et al., 2006; Wilkinson et al., 2006; Getachew and Gedamu, 2007; Bodył and Mackiewicz, 2008). The GFP-based experiments of Dufernez et al. (2006) and Wilkinson et al. (2006) also demonstrate that SODCs are delivered to the mitochondrial matrix, but their targeting to the intermembrane space is an interesting hypothesis that deserves further consideration.

Superoxide radicals cannot cross membranes easily and mitochondria possess superoxide dismutases in their intermembrane space (see, for example, Krumova et al., 2008). Thus, it is reasonable to assume that trypanosomatid mitochondria also have their own intermembrane space–located SODs. It was found that proteins imported into the intermembrane space in the Trypanosomatidae possess very short pre-sequences, e.g., Rieske protein (Priest and Hajduk, 1996), or are devoid of them as in the case of cytochrome c_1 (Priest and Hajduk, 2003). These data, however, do not exclude transport of SODCs into the intermembrane space. The N-terminal domains of trypanosomatid SODC pre-sequences have features of signal peptides and they could function as targeting signals for translocation across the inner mitochondrial membrane into the intermembrane space via a pathway known as the Sec translocon (for a review, see Driessen and Nouwen, 2008). This pathway operated in the α-proteobacterial ancestor of mitochondria (Gatsos et al., 2008) and is still preserved by some mitochondria (Dolezal et al., 2006). However, trypanosomatid mitochondria are devoid of a Sec translocation system (Schneider et al., 2008). Consequently, this route of protein trafficking into the intermembrane space seems to not be used by SODCs.

Considering the absence of Sec translocon in the trypanosomatid mitochondrial inner membrane, we propose an alternative model for SODC targeting to the intermembrane space. Because the N-terminal domains of SODC pre-sequences have a hydrophobic nature, it is possible that some percentage of these dismutases is still recognized by the signal

recognition particle (SRP) and targeted to the ER (for a model of such targeting, see Levitan et al., 2005; Bodył and Mackiewicz, 2007). After translocation into the ER lumen, they could move further via the same endomembrane pathway used by proteins targeted to the trypanosomatid plastid. However, currently, the ER- or Golgi apparatus–derived transport vesicles would not fuse with the outermost plastid membrane, but with the outer mitochondrial membrane, liberating SODCs directly into the intermembrane space. The remaining SODCs would be imported post-translationally into the mitochondrial matrix, using their whole pre-sequences as mitochondrial targeting signals.

PERSPECTIVES

It is usually assumed that the hypothetical trypanosomatid plastid evolved from the same green algal endosymbiont as the euglenid plastid (Fig. 2A) (Hannaert et al., 2003; Martin and Borst, 2003); however, there is no reason to exclude the possibility of their independent origins (Fig. 2B,D), as indicated by the case of $\Delta 4$ desaturases discussed in the Tertiary endosymbiosis subsection. Green alga–derived plastids occur in euglenids, chlorarachniophytes, and some dinoflagellates, such as *Lepidodinium viride* and *Gymnodinium chlorophorum* (Elbrächter and Schnepf, 1996; Rogers et al., 2007; Silver et al., 2007). It was hypothesized that euglenid and chlorarachniophyte plastids were derived from the same secondary endosymbiosis (Cavalier-Smith, 2003), but recent data clearly demonstrate their separate acquisitions from distinct green algal lineages (Rogers et al., 2007; Takahashi et al., 2007). Thus, at present, we have evidence for at least 3 independent green algal endosymbioses (in euglenids, chlorarachniophytes, and dinoflagellates) and it certainly is reasonable that one more such endosymbiosis could have happened in trypanosomatid parasites.

A direct test for the trypanosomatid plastid can be made through characterization of superoxide dismutases from photosynthetic euglenids like *E. gracilis*. If the euglenid plastid–directed SODs do not group with SODCs from *Trypanosoma* and *Leishmania* species on phylogenetic trees, it will indicate that trypanosmatid and euglenid plastids resulted from independent endosymbiotic events. If $\Delta 4$ desaturase phylogenies do, in fact, reflect an endosymbiotic gene transfer, the hypothetical trypanosomatid plastid could have evolved from a haptophyte or stramenopile alga via tertiary endosymbiosis. Thus, it would belong to the red, rather than the green, plastid lineage, in clear contrast with euglenid plastids. Red alga–derived plastids probably evolved by multiple endosymbioses (involving secondary, tertiary, and quaternary endosymbiotic events) (see Bodył et al., 2009), and this evolutionary scenario should be considered seriously for the Trypanosomatidae.

Additional studies of GFP localization should be undertaken using SODC pre-sequences. It will be interesting to determine whether some of them are still able to target GFP to complex plastids, such as those of euglenids and/or dinoflagellates.

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